

BRAF MUTATIONS IN SPORADIC COLORECTAL CARCINOMA FROM POLISH PATIENTS

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BRAF mutations are second to KRAS mutations in activation of the MAPK pathway in colorectal carcinoma cells. In addition to mutated KRAS, BRAF V600E mutation is associated with resistance to EGFR-targeted therapy in colorectal cancer; thus mutated BRAF might serve as a predictive factor. In this study, 163 routinely resected adenocarcinomas were screened for mutations in exons 11 and 15 of the BRAF gene. Only 6 (3.7%) tumours had a missense point mutation (G469A, D594G, G596R, K601N and twice V600E). The tumours were locally advanced with multiple metastases to lymph nodes. Mutations were associated with microsatellite instability (2 cases MSI-H, 2 cases MSI-L) and mutually exclusive with a mutated KRAS gene. In this sample set, mutations in the BRAF gene were more diverse and less frequent than usually reported.

Key words: BRAF, mutations, colorectal cancer.

Introduction

The BRAF gene encodes serine/threonine kinase activated by somatic point mutations in human cancers. K-Ras and B-Raf are involved in the transduction of mitogenic signals from the cell membrane to the nucleus. Recent clinical trials indicate that BRAF mutations are associated with resistance to anti-EGFR antibodies in colorectal cancers with wild-type KRAS [1-3]. Moreover, several drugs targeting B-Raf are in development or currently in clinical trials, including selective V600E-mutant inhibitors [4, 5]. Depending on therapeutic approach, BRAF mutations may serve as an exclusion or inclusion criterion; thus they constitute an important predictive factor. Allele-specific PCR is frequently in use for V600E mutation detection based on an assumption that this mutation is predominant; however, rare mutations in other codons are omitted.

Here we present the results of SSCP and sequencing analysis of exons 11 and 15 of the BRAF gene in routinely resected sporadic colorectal cancers previously screened for KRAS mutations.

Materials and methods

Samples

One hundred and sixty-three unselected sporadic colorectal carcinoma cases were studied. All the patients (91 men and 72 women, aged 34-87 years) underwent surgery at the 1st Department of Surgery, *Collegium Medicum*, Jagiellonian University, Kraków, Poland. Carcinomas were histologically staged according to the TNM classification. The tumour and normal mucosa samples were cut into small pieces, snap frozen and stored at -80°C for further investigation. DNA was extracted from fresh-frozen tumour and corresponding non-neoplastic tissues according to the manufacturer's instructions (QIAamp DNA Mini Kit, Qiagen).

Mutation detection

Mutational analysis of BRAF was performed by SSCP and sequencing. PCR products were amplified with RedTaq polymerase (Sigma-Aldrich) using the primers:

5'-TTCTGTTTGGCTTGACTTGACTT
5'-ACTTGTCAATGTCACCACATT
for BRAF exon 11 [6] and
5'-TCATAATGCTTGCTCTGATAGGA

5'-GGCCAAAAATTTAATCAGTGGA for BRAF exon 15 [7] (annealing temp. 57°C). Electrophoresis was carried out in TBE buffer (MP Biomedicals) using 80% MDE gels (Cambrex Bio Science). Thermostatically controlled circulating water was used to maintain a constant temperature of 8°C. Gels were run for 18 h with constant 170 V and developed by silver staining. Mutations were confirmed by direct sequencing. The sequence data were collected and analysed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). KRAS mutations were detected by SSCP and sequencing as described in detail previously [8].

MSI analysis

PCR was performed according to the previously reported protocol using a panel of 5 microsatellite markers located near APC, p53, BATRII, BAT26, and BAX [9]. All the cases that demonstrated MSI or LOH in at least 1 microsatellite marker were examined with a panel of 9 microsatellite markers (D2S123, D5S346, D3S1611, D18S35, NM-23, D7S501, D1S2883, TP53-pentanucleotide, TP53-dinucleotide) using a set of primers labelled with fluorescent dyes. The PCR products were separated on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). According to the MSI/LOH results, the cancers were classified into 3 groups: MSS (microsatellite stable cancers, without detectable instability), MSI-high (at least 40% unstable markers), and MSI-low [10].

Results

Mutational analysis performed on 163 sporadic colorectal adenocarcinoma cases revealed BRAF mutations in 6 tumours (3.7%). All of these mutations were missense. Five mutations were localized in exon 15: D594G, G596R, K601N and twice V600E. Only 1 mutation was detected in exon 11 (G469A).

Tumours with mutation in exon 15 were locally advanced (T3 or T4) at the time of resection. The only tumour with mutation in exon 11 was invading a muscular tissue (T2). All were metastatic to lymph nodes without diagnosed distant metastases. A BRAF mutation was detected in only 2 of 14 MSI-H cancers (Fisher's test MSI-H vs. MSS/MSI-L; $p = 0.08$). These 2 mutated tumours with a high level of microsatellite instability had typical right-sided location and moderate or poor differentiation. Four others were situated distally to the splenic flexure with stable microsatellites (MSS) or a low level of instability (MSI-L) and moderate to poor differentiation. No mutation was detected in well differentiated tumours, which made up 25% (41/163) of the specimens. Tumours with V600E as well as exon 11 mutations were poorly differentiated. Mucus production characterized 19% (31/163) of collected adenocarcinomas and none of them carried a BRAF mutation whereas 45% (14/31) had a KRAS mutation. Mutated KRAS was detected in 36% (58/163) of the specimens [8]. The BRAF and KRAS mutations were mutually exclusive.

Discussion

Oncogenes BRAF and KRAS play a role in increased activity of the MAPK pathway, which is involved in cancer cell proliferation and migration. Due to mutations, altered B-Raf kinase may influence therapy outcome as it does in EGFR inhibition refractory colorectal cancers. BRAF mutation testing can provide useful information for planning therapy for individual patients. The reported frequency of mutations varies in sporadic colorectal cancers between 3 and 21% (Table II). In our study, the incidence was one of the lowest in unselected samples. A mutation was observed only in 3.7% of the collected tumours.

Allele-specific PCR is usually employed for detection of the most common mutation in codon 600. Interestingly, V600E mutation was not predominant in this sample set: only 2 out of 6 mutations (33%). Yuen *et al.* reported a similar

Table I. Tumours with BRAF mutation

MUTATION	LOCATION	MSI STATUS	TNM	DIFFERENTIATION
p.G469A (c.1406G>C)	Rectum	MSS	T2N1M0	Poor
p.D594G (c.1781A>G)	Rectum	MSI-L	T4N2M0	Moderate
p.G596R (c.1786G>C)	Right colon	MSI-H	T3N2M0	Moderate
p.V600E (c.1799T>A)	Right colon	MSI-H	T3N2M0	Poor
p.V600E (c.1799T>A)	Left colon	MSS	T3N2M0	Poor
p.K601N (c.1803A>T)	Left colon	MSI-L	T3N2M0	Moderate

TABLE II. BRAF MUTATION FREQUENCIES IN UNSELECTED SPECIMENS OF COLORECTAL CANCER

STUDY [REFERENCE]	NO. OF CASES	% (NO.) WITH BRAF MUTATION	GENE FRAGMENT TESTED
Miranda <i>et al.</i> 2006 [11]	202	3.5 (7)	V600E only
Maestro <i>et al.</i> 2007 [12]	324	3.7 (12)	V600E only
Lee <i>et al.</i> 2008 [13]	134	4.5 (6)	V600E only
Asaka <i>et al.</i> 2009 [14]	908	4.5 (41)	V600E only
Yuen <i>et al.</i> 2002 [15]	215	5.1 (11)	Exons 11 and 15
Suehiro <i>et al.</i> 2008 [16]	199	6.5 (13)	Exons 11 and 15
Ikehara <i>et al.</i> 2005 [17]	82	7.3 (6)	Exons 11 and 15
Nagasaka <i>et al.</i> 2004 [18]	234	9.0 (21)	V600E only
Samowitz <i>et al.</i> 2005 [19] *	911	9.5 (87)	V600E only
Rajagopalan <i>et al.</i> 2002 [20]	330	9.7 (32)	Exons 11 and 15
Deng <i>et al.</i> 2004 [21]	80	10.0 (8)	V600E **
Shen <i>et al.</i> 2007 [22]	87	12.6 (11)	Exons 11 and 15
Barault <i>et al.</i> 2008 [23] *	585	13.3 (78)	V600E only
Weisenberger <i>et al.</i> 2006 [24]	187	13.9 (26)	V600E only
Ogino <i>et al.</i> 2006 [25]	837	14.1 (118)	V600E only
Goel <i>et al.</i> 2007 [26]	126	20.6 (26)	V600E only

* colon cancers only; ** mutation-specific PCR for V600E detection in 65 tumours and sequencing of 2 exons in 15 tumours.

Table III. Studies with exons 11 and 15 analysis of BRAF gene in colorectal cancer

STUDY [REFERENCE]	FREQUENCY OF V600E IN BRAF MUTATION CASES (%)	FREQUENCY OF EXON 11 MUTATIONS IN BRAF MUTATION CASES (%)
Yuen <i>et al.</i> 2002 [15]	36	18
Lubomierski <i>et al.</i> 2005 [27]	50	14
Suehiro <i>et al.</i> 2008 [16]	69	15
Ahlquist <i>et al.</i> 2008 [28]	79	6
Ikehara <i>et al.</i> 2005 [17]	83	17
Rajagopalan <i>et al.</i> 2002 [20]	88	9
Wang <i>et al.</i> 2003 [29]	95	2

proportion (36%) [15]. Some authors have analysed 2 exons of the BRAF gene. The percentage of V600E mutations varied between 36% and 95% of the BRAF mutations in such studies (Table III). In our study, exon involvement resembled already published data. Five mutations in exon 15 (codons 594–601) and only 1 in exon 11 (codon 469) were observed. Mutations in exon 11 occurs rarely. Depending on the cohort studied, the percentage of mutations in exon 11 fluctuated between 2% and 18% of all BRAF mutations (Table III).

Many authors have reported a high incidence of BRAF mutations in MSI-H cancers: Maestro *et al.* 18.4% [12], Lubomierski *et al.* 27% [27], Rajagopalan *et al.* 30.6% [20], Asaka *et al.* 32% [14], Nagasaka *et al.* 45% [18], Samowitz *et al.* 52% [19], Ahlquist *et al.* 62% [28], Barault *et al.* 63% [23], Goel *et al.* 71% [26]. The association with

a high level of microsatellite instability is well established. However, in the present study, BRAF mutations were relatively rare in MSI-H cancers (14%). The trend towards higher incidence in this group is statistically insignificant in comparison with MSS. No BRAF mutation has been detected in well differentiated carcinomas. An association of mutated BRAF with poor differentiation is sometimes reported [18, 19]. Contrary to published data, none of the 6 tumours with mutation had mucinous histology [19, 30, 31].

Oncogenic mutation in either KRAS or BRAF is usually sufficient for dysregulation of the MAPK pathway in a cancer cell. Coexisting mutations in both genes are rare findings [15, 19]. Our results support this observation. No tumour with mutated BRAF had concomitant mutated KRAS.

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